Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma

TATSUHIKO IGARASHI[1], CHARLES BROWN[1], ALI AZADEGAN[2], NANCY HAIGWOOD[3], DIMITER DIMITROV[4], MALCOLM A. MARTIN[1] & RIRI SHIBATA[1]

[1]*Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA*
[2]*Veterinary Resource Program, National Institutes of Health, Bethesda, Maryland 20892, USA*
[3]*Seattle Biomedical Research Institute, Seattle, Washington 98109, USA*
[4]*Laboratory of Experimental and Computational Biology, National Cancer Institute, National Institutes of Health, Frederick, Maryland 21702, USA*
R.S. present address: VaxGen, 1000 Marina Boulevard, Brisbane, California 94005-1841, USA

The concentration of human immunodeficiency virus type 1 (HIV-1) particles in blood plasma is very predictive of the subsequent disease course in an infected individual; its measurement has become one of the most important parameters for monitoring clinical status[1]. Steady-state virus levels in plasma reflect a balance between the rates of virions entering and leaving the peripheral blood[2]. We analyzed the rate of virus clearance in the general circulation in rhesus macaques receiving a continuous infusion of cell-free particles in the presence and absence of virus-specific antibodies. Here we show, by measuring virion RNA, particle-associated p24 Gag protein and virus infectivity, that the clearance of physical and infectious particles from a primary, dual-tropic virus isolate, HIV-1_{DH12}, is very rapid in naive animals, with half-lives ranging from 13 to 26 minutes. In the presence of high-titer HIV-1_{DH12}-specific neutralizing antibodies, the half-life of virion RNA was considerably reduced (to 3.9–7.2 minutes), and infectious virus in the blood became undetectable. Although physical virus particles were eliminated extravascularly, the loss of virus infectivity in the blood reflected the combined effects of extravascular clearance and intravascular inactivation of HIV-1 infectivity due to antibody binding.

We have examined the clearance rates of physical and infectious HIV-1 particles in macaque monkeys and the influence of virus-specific antibodies on these rates using a variety of sensitive detection methods, well-characterized HIV-1 and chimeric simian-human immunodeficiency virus[3] (SHIV) strains and different subhuman primate model systems. Rhesus monkeys (*macaca mulatta*) were the principal macaque species used (Table) because they do not support a productive HIV-1 infection although the virus does, in fact, bind to and enter macaque CD4-positive T lymphocytes[4,5]; and they can be persistently infected with SHIVs carrying an HIV-1-derived envelope and produce neutralizing antibodies directed against the envelope glycoproteins associated with both the SHIV as well as the parental strain of HIV-1 (ref. 6). Two pig-tailed monkeys, a macaque species reported to be marginally susceptible to HIV-1 infection[7], were also used (Table). We slowly infused an HIV-1 suspension into macaque monkeys and then monitored virus clearance from the blood over a period of 102–290 minutes. The highly cytopathic, rapidly-replicating and T-cell/macrophage dual-tropic primary isolate HIV-1_{DH12} (ref. 8)(Fig. 1*a*) was selected for infusion because a high-titer, cell-free virus stock could readily be prepared in human peripheral blood mononuclear cells (PBMC); rhesus macaques chronically infected with SHIVs with homologous (HIV_{DH12}) or heterologous (HIV-1_{HXB2}) envelope glycoproteins were available[9,10] (Fig. 1*a*); and a large quantity of IgG, capable of neutralizing HIV-1_{DH12}, was available from chimpanzees persistently infected with HIV-1_{DH12} (ref. 8).

The virus stock used for all clearance studies in macaques was prepared by infecting human PBMC with virus derived from a molecular clone of HIV-1_{DH12} (called DH123) in which all nine open reading frames were open[8]. HIV-1_{DH12} (10 ml, containing 4.2×10^8 $TCID_{50}$ (50% tissue culture infectious dose), 2820 ng p24 Gag and 7.5×10^9 RNA copies) was resuspended in 90 ml of Ringer's solution and infused intravenously into anesthetized animals over a period of 20 to 190 minutes. During and after the HIV-1 infusion, samples of whole blood were collected at 10- to 15-minute intervals, and the separated plasma was analyzed for viral RNA, p24 Gag proteins and virus infectivity in MT-4 cells.

We first monitored the half-life of HIV-1 in naive rhesus macaques. The plasma viral RNA level during the 'loading' phase was relatively constant in naive animal 93B027 (Fig. 2*a*, left). After HIV-1 infusion, the viral RNA concentration declined rapidly with a half-life of 19.3 minutes. Similar virion RNA half-lives (15.9 and 13.0 minutes; Fig. 2*a*) were measured in two other naive rhesus macaques (93B042 and 93B047), even though the steady-state plasma RNA levels at the end of the infusion phase varied (approximately 1.5×10^5, 5×10^4 and 2×10^5 RNA copies/ml, respectively). These differences presumably reflected different rates of HIV-1 infusion into the three rhesus monkeys. To confirm the results obtained by monitoring virion RNA levels, we analyzed the same set of samples using two other parameters. The half-lives of pelletable plasma p24, presumably representing virion-associated Gag p24, were 13.4, 11.2 and 12.0 minutes in the same three naive animals (Fig. 2*b*). The half-lives of 'free' p24 in the plasma of these animals were substantially longer (27.9, 22.3 and 29.1 minutes; Fig. 2*b*), most likely reflecting the effect(s) of particle size on clearance from the blood[11] and/or the continuous shedding of p24 from circulating virus particles. The third parameter, HIV-1 infectivity in MT-4 cells[8], was measured in quadruplicate assays by end-point titration of

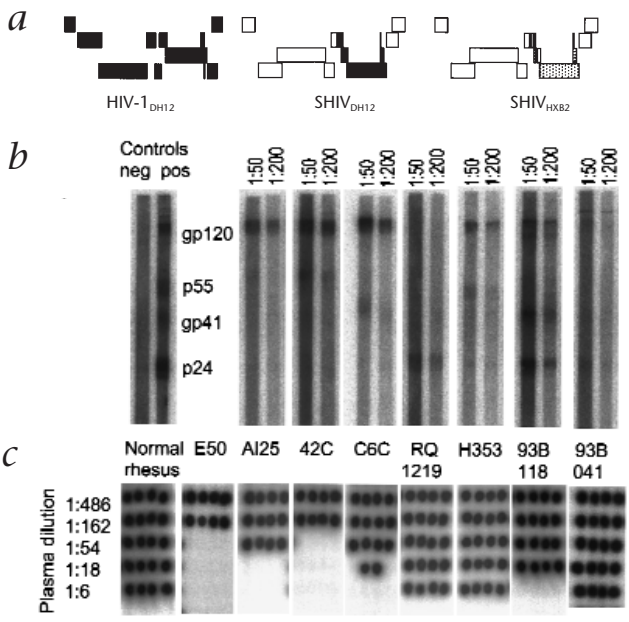


Fig. 1 ***a***, Genomic structures of HIV-1 and SHIVs used here. Binding (*b*) and neutralizing (*c*) antibodies directed against HIV-1_{DH12} present in the plasma of persistently infected or passively immunized macaques. Rhesus monkeys E50, AI25, 42C and C6C were persistently infected with $SHIV_{DH12}$ (ref. 9), and animals RQ1219 and H353 were chronically infected with $SHIV_{HXB2}$ (ref. 10). Macaques 93B118 and 93B041 were passively immunized with 2.0 g and 0.2 g, respectively, of IgG purified from a chimpanzee persistently infected with HIV-1_{DH12} and two other primary isolates, 24 h before virus infusion. ***b***, Plasma samples were diluted to 1:50 and 1:200 for immunoblotting using an HIV-1_{DH12} infected PBMC lysate as antigen. The envelope glycoproteins (gp120 and gp41) and viral core proteins (Gag p55 and p24) are indicated.Controls: neg, plasma from uninfected monkey; pros, plasma from HIV-1 infected chimpanzee. ***c***, Virus neutralization assays; 100 $TCID_{50}$ of HIV-1_{DH12} were incubated with serially diluted plasma samples and used for quadruplicate infections of MT-4 cells[25]. In autoradiograms of ^{32}P reverse transcriptase assays done 2 weeks after infection, positive virus replication (black dots) indicates no neutralization.

fourfold serial dilutions of plasma, collected at various times during the loading or clearance phases of the experiment. The biological half-lives of the infused HIV-1_{DH12}, as monitored by $TCID_{50}$, were 19.8, 18.9 and 25.8 minutes in the three naive macaques (Fig. 2*c*). In these and other estimates of HIV-1 clearance, linear regression curves were generated based on the levels of viral RNA, pelletable p24 or $TCID_{50}$ determined during the 60-minute period immediately after virus infusion.

Although the clearance values measured in the three assays differed somewhat, they were all in a range similar to that reported for the half-life of Langat virus, a flavivirus studied in spider monkeys[2]. Two additional HIV clearance experiments were done in naive pig-tailed macaques, and similar half-life values were obtained (Table). These data show that the clearance of HIV-1 virions from the blood of naive monkeys is very rapid. Because HIV-1-infected individuals produce virus-specific antibodies, we next examined the possible role of such antibodies in the elimination of virus from the general circulation.

We studied three groups of animals with qualitatively and quantitatively different antibodies directed against HIV-1_{DH12} (Table). Animals C6C, 42C, AI25 and E50 had been persistently infected with the nonpathogenic $SHIV_{DH12/MD1}$ (ref. 9) for 2–3 years (Fig. 1*a*). All four had binding (Fig. 1*b*; animal E50 not shown) and neutralizing (Fig. 1*c*) antibodies directed against HIV-1_{DH12}. Animals RQ1219 and H353 had been persistently infected for 1.5 years with $SHIV_{HXB2}$ (ref. 10)(Fig. 1*a*), a chimeric virus bearing HIV-1_{HXB2} *env* sequences. Antibodies binding to the HIV-1_{DH12} Env protein were detected in the plasma of macaque H353 and to p24 Gag in the sample from animal RQ1219 (Fig. 1*b*). However, neither of these plasma samples neutralized HIV-1_{DH12} (Fig. 1*c*), consistent with the lack of cross-neutralization of HIV-1_{DH12} and HIV-$1_{IIIB/HXB2}$ with sera from chimpanzees infected with each of these HIV-1 isolates[12]. Animals 93B118 and 93B041 had never been infected with SHIV or SIV, but were passively immunized, 24 hours before receiving the HIV-1 infusion, with IgG purified from an HIV-1 seropositive chimpanzee that had developed neutralizing antibodies against HIV-1_{DH12} (R.S. and M.A.M., unpublished data). At the time of virus infusion, binding (Fig. 1*b*) and neutralizing (Fig.1*c*) antibodies were evident in macaque 93B118, which had been passively immunized with 2 g (330 mg/kg) of the chimpanzee IgG. Weak binding activity to p24 Gag and no neutralizing activity was detectable in macaque 93B041, which had received tenfold less (0.2 g; 40 mg/kg) chimpanzee IgG (Fig. 1*b* and *c*). These plasma neutralizing and binding antibody levels are summarized in the Table.

Assessment of HIV-1 clearance in the four macaques, which were persistently infected

Table Animal characteristics

Animal ID	Body weight (kg)	Persistent infection	Passive immunization	Binding antibody env	Binding antibody Gag	Neut antibody titer	Half-life of plasma HIV-1 (minutes) Virion RNA	Pelletable Gag p24	Infectivity
93B027	5.9	none	none	NT	NT	NT	19.3	13.4	19.8
93B042	5.4	none	none	NT	NT	NT	15.9	11.2	18.9
93B047	6.2	none	none	NT	NT	NT	13.0	12.0	25.8
1001 (pt)	6.2	none	none	NT	NT	NT	13.5	16.8	NT
093P (pt)	5.4	none	none	NT	NT	NT	13.9	24.8	NT
C6C	6.5	$SHIV_{DH12}$	none	169	<30	1:18	7.2	NT	Undetectable
42C	8.5	$SHIV_{DH12}$	none	306	32	1:93	3.9	NT	Undetectable
AI25	6.1	$SHIV_{DH12}$	none	115	<30	1:31	3.9	NT	Undetectable
E50	8.7	$SHIV_{DH12}$	none	NT	NT	1:93	3.9	NT	Undetectable
93B041	5.0	none	0.2 g	<30	35	<1:6	23.9	NT	8.7
93B118	6.1	none	2.0 g	110	94	1:10	6.7	NT	Undetectable
RQ1219	7.2	$SHIV_{HXB2}$	none	<30	72	<1:6	31.2	NT	29.6
H353	5.8	$SHIV_{HXB2}$	none	63	<30	<1:6	12.6	NT	10.3

Eleven rhesus macaques and two pig-tailed macaques (pt) were used here. Six animals were persistently infected with two different SHIVs (Fig. 1*a*) for more than 1 year and showed no clinical symptoms. Two of the macaques (93B041 and 93B118) were passively immunized 24 h before virus infusion with IgG purified from an HIV-1 seropositive chimpanzee. The chimpanzee IgG donor (4749) had been infected by transfusing whole blood from another chimpanzee (Ch4750), persistently infected with HIV-1_{DH12}, and two other HIV-1 primary isolates (HIV-1_{DH20} and HIV-$1DH_{29}$ (ref. 23)). IgG was purified as described[24]. The half-life of chimpanzee IgG in macaques is 10–14 days (not shown). Binding activities against HIV-1_{DH12} proteins were determined by phosphoimager analysis of the radioactivity associated with gp120 Env or p24 Gag proteins in the immunoblotting analysis shown in Fig. 1*b* (1:200 dilution). Neutralization titers[25] against 100 $TCID_{50}$ of HIV-1_{DH12} are shown (Fig. 1*c*). NT, not tested.

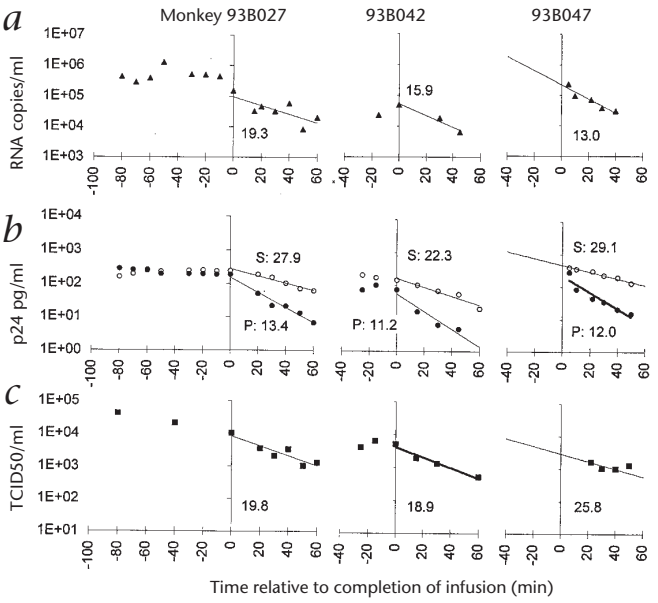


Fig. 2 Clearance of physical and infectious HIV-1 particles in three naive rhesus macaques. ***a***, Plasma RNA was quantified by RT–PCR as described[25]. ***b***, For the measurement of p24 Gag protein, plasma samples were centrifuged and the pellet (P) and supernatant (S) fractions were assayed by an antigen capture ELISA assay. ***c***, HIV-1 infectivity in plasma was measured by end-point titration using MT-4 cells in a 14-day assay[8]. The half-lives (in minutes) were calculated from the theoretical curves shown by solid lines; separate curves and decay rates were determined for pelleted (P) and soluble (S) p24 Gag protein determinations.

with $SHIV_{DH12}$ and produced neutralizing antibodies directed against $HIV\text{-}1_{DH12}$, showed that none had detectable levels of infectious virus during or after HIV-1 infusion (Fig. 3*a*). In animals 42C and C6C, for example, no infectious virus was detectable in the plasma even during the first 20 minutes of virus infusion (administered at a rate of 2.2×10^6 $TCID_{50}$/minute and 3.5×10^6 $TCID_{50}$/minute, respectively), a result indicating that neutralization of HIV-1 infectivity *in vivo* is very rapid. Although virion RNA was detectable by RT–PCR in plasma during or after virus infusion in these four animals (Fig. 3*b*), virion clearance was considerably accelerated (their half-lives were reduced to 3.9–7.2 minutes) compared with the values in the naive monkeys (Fig. 2*a*; 13.0–19.3 minutes). A similar phenomenon was seen in the two animals passively immunized with the chimpanzee IgG. Acceleration of both the infectivity and virion RNA clearance rates was observed and found to be dependent on the amount of neutralizing IgG passively transferred to each recipient. The half-life of infectious virions in animal 93B118, passively immunized with the highest dose of IgG (2 g), was not measurable, whereas in animal 93B041, which received the low IgG dose (0.2 g), the biological virus half-life was 8.7 minutes (Fig. 4*a* and *b*). The half-life of virion RNA in 93B118 was 6.7 minutes, and that in 93B041 was longer (23.9 minutes). Virus clearance, as measured by RT–PCR or infectivity, in the two $SHIV_{HXB2}$ infected rhesus macaques (H353 and RQ1219), which had binding but no neutralizing antibodies against $HIV\text{-}1_{DH12}$, was similar to that seen in the naive monkeys (Figs. 4*a* and *b*).

To quantitatively assess the statistical significance of the effect of virus-specific neutralizing antibodies on HIV-1 clearance, we divided the animals into two groups based on whether they had detectable (titer > 1:6) or undetectable neutralizing antibodies (Table). The first group included five animals (C6C, 42C, A125, E50 and 93B118), and the second, eight macaques (93B027, 93B042, 93B047, 1001, 093P, 93B041, RQ1219 and H353). The virus decay rate constants for each group were calculated from the HIV-1 RNA levels measured during the first 40–45 minutes after infusion, because they provided the highest correlation coefficients (on average, $R = 0.92$, corresponding to $P = 0.027$ for each animal) between the observed and fitted values. The mean virus decay rate constant (0.16 ± 0.02 per minute) in macaques with detectable neutralizing antibodies was significantly higher ($P = 0.000003$) than that calculated for animals with no neutralizing antibodies (0.057 ± 0.019 per minute). To further evaluate the significance of the difference between the two groups of animals and to minimize the effects of inter-animal variability, we normalized the viral RNA values for each animal at the conclusion of HIV-1 infusion, and subjected the logarithms of all subsequent time points to linear regression analysis (Fig. 4*c*). The overall virus decay rate constants calculated after normalization (0.15 per minute and 0.060 per minute for neutralizing antibody-positive and -negative animals, respectively) correspond to clearance half-lives of 4.5 and 11.5 minutes, which is similar to the average values of the rate constants for individual animals in each group. The mean HIV RNA concentrations for the two groups of animals were significantly different ($P = 0.000001$). In some of the animals from both groups, for which later data points were available, a much slower second phase of HIV-1 clearance was observed.

We also investigated the mechanism(s) responsible for clearance of physical and infectious virus particles by simultaneously comparing the half-lives of HIV-1 *in vivo* and *ex vivo*. After HIV-1 was infused into animal 93B118 (the recipient of the larger amount (2.0 g) of neutralizing chimpanzee IgG), a 15-ml sample of blood was collected, treated with anticoagulant and maintained at body

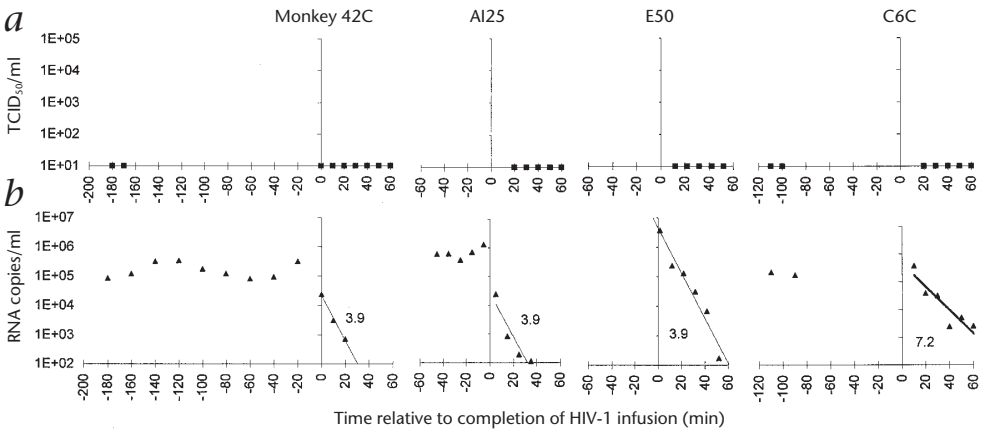


Fig. 3 Clearance of HIV-1 in rhesus macaques persistently infected with $SHIV_{DH12}$ that produce neutralizing antibodies directed against $HIV\text{-}1_{DH12}$ gp120. Infectivity (***a***) and levels of virion RNA (***b***) were measured.

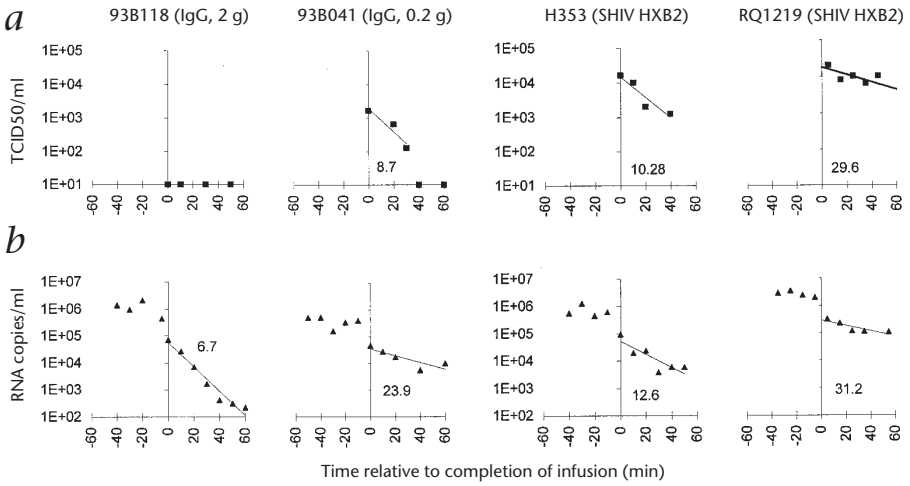
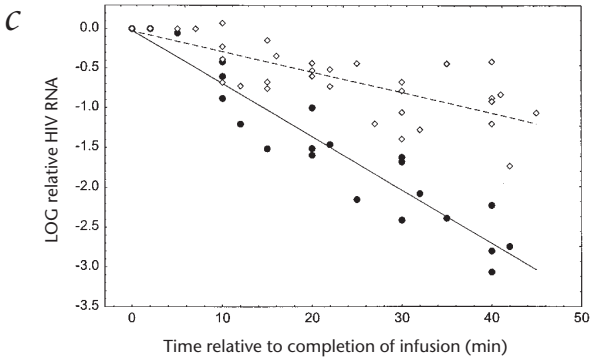


Fig. 4 The clearance of HIV-1 is accelerated *in vivo* in the presence of neutralizing antibodies. Infectivity (***a***) and virion RNA levels (***b***) were measured in rhesus macaques after passive transfer of the indicated amounts of chimpanzee IgG (93B118 and 93B041) or in animals chronically infected with $SHIV_{HXB2}$ (H353 andRQ1219). Neutralizing antibody titers in the plasma of these four macaques are shown in Fig. 1*c*. ***c***, The logarithms of plasma HIV RNA concentrations, determined at approximately 10-min intervals after virus infusion ended for all animals listed in the Table, were normalized to their initial values and plotted. ●, animals with detectable neutralizing antibodies (titer > 1:6); ◇, animals with no detectable neutralizing antibodies. Solid and dashed lines represent the least square fits to the experimental points for the two groups. The slopes of the two lines are 0.067 per min and 0.026 per min, corresponding to average virus decay rate constants of 0.154 per min and 0.060 per min, and half-lives of 4.5 and 11.5 min, respectively.



temperature (the *ex vivo* specimen). Samples were then collected every 10 minutes from this tube (*ex vivo* samples), a model of possible intravascular virus degradation, and the saphenous vein of macaque 93B118. Thus, we simultaneously measured intra- and extravascular clearance of HIV-1, respectively. The levels of viral RNA in the *ex vivo* sample remained relatively constant, whereas virus present in the circulating blood (the *in vivo* sample) during this period was rapidly cleared (Fig. 5*a*). In contrast, no infectious virus particles were detected in either the *ex vivo* or *in vivo* samples, because of the presence of neutralizing antibody (Fig. 5*b*).

These results demonstrate that the observed rapid elimination of physical virus particles *in vivo* reflects extravascular clearance, not intravascular virion degradation. Indeed, the reticuloendothelial (RE) system and/or the mononuclear phagocyte system (for example, Kupffer cells in the liver) are responsible for the rapid clearance of cell-free virions from the blood[13]. *In situ* hybridization studies of liver, spleen, lymph node and kidney specimens, collected postmortem within 2 to 4 hours of virus infusion, failed to demonstrate the presence of any HIV-1 RNA. This probably reflects the rapid degradation of cleared virus in the RE system and/or our inability to detect the systemically distributed virus inoculum (7.5×10^9 HIV-1 RNA molecules) initially administered.

Here we have essentially revisited a series of experiments from the 1950s and 1960s[2,13–16] that examined the rapid elimination of cell-free virions from circulating blood. Our data extend these observations to HIV-1, which is also cleared extremely rapidly. Our experiments only measured the decay rate of virions present in the blood in the absence of any *de novo* virus production. In studies of the decay rate of plasma virus RNA in AIDS patients after the administration of potent antiviral drugs, the measured virion RNA levels declined with half-lives ranging from 1.5 to 3 days[17,18]. The decline of the steady-state HIV-1 levels in infected individuals reflects both the intrinsic clearance of virions already in the blood as well as the diminishing amounts of progeny virus particles entering the circulation as a consequence of potent antiviral therapy. A re-analysis of the original data using a different mathematical model estimated the half-life of virions to be 6 hours[19]. This value contrasts with our HIV-1 half-life value in macaque monkeys (13–26 minutes), based on a direct measurement of the decay of physical and biological virus particles.

The most straightforward interpretation of our results is that neutralizing antibody and not the binding, non-neutralizing antibody directed against HIV-1 accelerated the clearance of virus from the blood. However, animal H353, which had been persistently infected with $SHIV_{HXB2}$, had a slightly faster clearance rate. In contrast to the second chronically $SHIV_{HXB2}$ infected macaque (RQ1219), the plasma of animal H353 contained antibody reactive with $HIV\text{-}1_{DH12}$ gp120 (Fig. 1*b*). Perhaps the role of this type of gp120 binding antibody in virus clearance can be clarified in future passive transfer experiments.

Among the animals studied, the four monkeys chronically infected with $SHIV_{DH12}$ may 'model' HIV-infected individuals with circulating antibodies directed against their own virus. The aver-

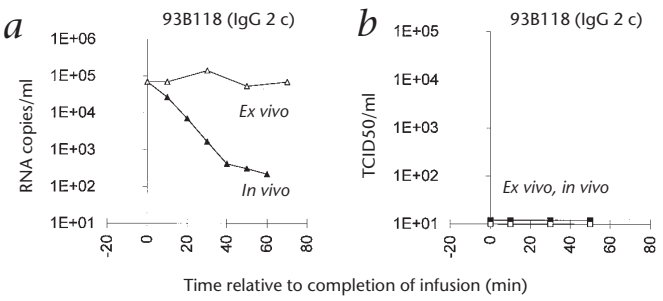


Fig. 5 *In vivo* and *ex vivo* clearance of HIV-1 physical particles in blood. Samples were obtained either from the saphenous vein (*in vivo*) or from a sample tube containing blood collected immediately after virus infusion (time 0) and maintained at body temperature (*ex vivo*). Virion RNA levels (***a***) and HIV-1 infectivity (***b***) were measured.

age half-life of virion RNA in these four animals was only 4.7 minutes. This indicates that in persistently infected humans, the cell-free virions present in plasma are the most recent progeny released from infected cells and represent the dominant viral quasi-species replicating at that point in time. The very short half-life of the plasma virus measured here also indicates the tremendous quantities of progeny virions that must be continuously produced to maintain relatively constant levels of viremia. In two of the four $SHIV_{DH12}$-infected animals, 42C and AI25, a steady-state level of virus was established during virus infusions lasting 190 and 65 minutes and at average RNA copy numbers of 1.8×10^5 and 7.1×10^5/ml, respectively. This is similar to the levels measured in HIV-1-infected individuals. Because the virus infusion rates for these animals were 3.9×10^7 and 11.5×10^7 RNA copies/minute (a total of 7.5×10^9 RNA copies divided by the total infusion time), the steady-state levels observed in the two macaques would be equivalent to approximately 5.6×10^{10} and 1.6×10^{11} virions released into the blood each day (the infusion rate × 60 minutes × 24 hours). For a 60-kg human, these values would be equivalent to as many as 3.9×10^{11} and 1.6×10^{12} HIV-1 particles entering the blood daily (Table shows animal body weights), an estimate similar to that reported (and revised)[17–19].

Our results demonstrate that virus-specific neutralizing antibodies facilitate the clearance of physical and infectious particles from circulating blood. However, this experimental system was designed to evaluate the effect of antibodies on the fate of HIV-1 virions already released into the circulating blood, only one of the many steps of the virus life cycle *in vivo* that could be targeted by such antibody. The effect of HIV-1 antibodies on other phases of the virus life cycle is also being studied now.

Methods

Animals. Rhesus macaques (*Macaca mulatta*), pig-tailed macaques (*Macaca nemestrina*) and a chimpanzee (*Pan troglodytes*) were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*[20] and were housed in a biosafety level 2 facility; biosafety level 3 practices were used. Animals were anesthetized with Tiletamine-HCl and Zolazepam-HCl (Telazol; Fort Dodge Laboratories, Fort Dodge, Iowa) for all procedures, including virus infusions and phlebotomies. Cannulas (I-CATH; DELMED, Canton, Massachusetts) were inserted bilaterally into the saphenous veins of each leg; one was used for virus infusion, and the other, for the collection of venous blood samples. During and after the HIV-1 infusion, 1- to 2-ml samples of whole blood were collected at 10- to 15-min intervals in tubes containing EDTA. Plasma was immediately separated by centrifugation, frozen on dry ice and stored at –80 °C. Chimpanzee IgG was administered intravenously into two rhesus macaques (93B041 and 93B118) 24 h before HIV-1 infusion.

HIV-1 stock used in virus clearance studies. The clade B, primary, dual-tropic isolate $HIV\text{-}1_{DH12}$ used here had been isolated from a US AIDS patient in the early 1990s (ref. 8). A proviral molecular clone, called $HIV\text{-}1_{DH123}$, was obtained from $HIV\text{-}1_{DH12}$-infected PBMC; an initiation codon was subsequently introduced at the beginning of Vpu coding sequences[8]. Human $CD4^+$ MT-4 cells were electroporated with $HIV\text{-}1_{DH123}$ proviral DNA using a gene pulser (Bio-Rad, Hercules, California) at 960 mF/0.3 kV, cultured for several days, and a cell-free culture supernantant with high infectivity was used to inoculate 30 million PHA-stimulated human PBMC at approximately 0.01 MOI (mutiplicity of infection). Supernatants from the infected human PBMC were collected daily between days 3 and 11. Samples with high virion-associated reverse transcriptase (RT) activities were 'pooled' (130 ml total volume), divided into aliquots (10 ml each) and stored at –80 °C. Each 10-ml aliquot of the $HIV\text{-}1_{DH12}$ stock contained a total of 4.2×10^8 $TCID_{50}$ (50% tissue culture infectious dose), 2820 ng Gag p24 and 7.5×10^9 RNA copies.

Viruses present in persistently infected animals. Four rhesus macaques (C6C, 42C, AI25 and E50) had been infected for 2–3 years with a nonpathogenic $SHIV_{DH12}$, called $SHIV_{DH12}$ clone MD1 (ref. 9), which contained a portion of the U3 LTR, the R-U5 LTR, gag, pol, vif and vpx, and the first 17 codons of vpr of SIV_{mac239} (ref. 21). The tat, rev, env and nef genes, the remainder of vpr and a portion of U3 LTR were derived mostly from $HIV\text{-}1_{DH12}$, except for small segments at SIV–HIV-1 junctions (145 bp in vpr; 27 bp in nef) that were of $HIV\text{-}1_{NL4\text{-}3}$ (ref. 22) origin. The $SHIV_{DH12/MD1}$ stock had been prepared in macaque monkey PBMC, and the macaque recipients had never been exposed to human-cell-derived reagents.

Two rhesus macaques (RQ1219 and H353) had been infected with an SHIV carrying the tat, rev and env genes derived from an $HIV\text{-}1_{IIB\ (HXB2)}$ isolate[10]. None of the six chronically SHIV-infected monkeys had detectable levels of plasma SHIV RNA at the time of infusion experiments.

The IgG donor chimpanzee (Ch 4749) had been transfused with whole blood from chimpanzee Ch 4750, infected with three clade B primary isolates $HIV\text{-}1_{DH12}$, $HIV\text{-}1_{DH20}$ and $HIV\text{-}1_{DH29}$ as described[23]. Highly specific DNA PCR demonstrated the presence of all three HIV-1 isolates (R.S., unpublished observation). IgG was prepared from chimpanzee plasma as described[24].

Antibody assays. Antibody levels in persistently infected and passively immunized animals were measured by immunoblotting, a commercial ELISA kit (Vironostika HIV-1 Microelisa System; Organon Teknika, Durham, North Carolina) and neutralization assays (100% neutralization against 100 $TCID_{50}$ of $HIV\text{-}1_{DH12}$,) as described[25].

Assays of HIV-1 clearance. The decay of HIV-1 infectivity (50% tissue culture infectious dose, $TCID_{50}$) during and after virus infusion was determined by quadruplicate infections of human $CD4^+$ MT-4 cells with fourfold serially diluted plasma. The end-point of virus infectivity was detected by ^{32}P-reverse transcriptase assay[26] 2 weeks after infection; $TCID_{50}$ was calculated as described by Reed and Muench[27]. The concentration of p24 Gag antigen in plasma was determined using a commercial kit (Coulter HIV-1 p24 Ag assay; Coulter, Miami, Florida). To separate virion-associated p24 from free p24 protein, 200 µl plasma was centrifuged at 14,000 rpm for one hour in a refrigerated microcentrifuge (Eppendorf S402; Brinkman Instruments, Westbury, New York). Virion RNA levels were determined either by branched DNA (ref. 28) or quantitative RT–PCR (ref. 25).

***Ex vivo* and *in vivo* blood samples.** After the infusion of HIV-1 into animal 93B118, a 15-ml sample of blood was collected, treated with anticoagulant, placed in a conical test tube, wrapped in gauze and maintained at body temperature by taping it to the animal's perineum (the *ex vivo* specimen). Samples were then collected every 10 minutes from this the tube (*ex vivo* samples) and the saphenous vein of macaque 93B118. The levels of viral RNA in these samples were then determined.

Acknowledgments

We thank G. Coleman for assistance in the animal experiments; M. Eckhaus and G. Miller for pathological analyses; C. Pierce for purification of immunoglobulin; M.G. Lewis for providing $SHIV_{HXB2}$ infected monkeys; and R. Dewar for bDNA assays.

RECEIVED 24 NOVEMBER; ACCEPTED 17 DECEMBER 1998

1. Mellors, J.W. *et al.* Prognosis in HIV-1 predicted by the quantity of virus in plasma. *Science* **272**, 1167–1170 (1996).
2. Nathanson N. and Harrington B. Experimental Infection of Monkeys with Langat Virus. II. Turnover of Circulating Virus. *Am. J. Epidemiol.* **85**, 494–502 (1967).
3. Shibata, R. *et al.* Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J. Virol.* **65**, 3514–3520 (1991).
4. Shibata, R., Sakai, H., Kawamura, M., Tokunaga, K. & Adachi, A. Early replication block of human immunodeficiency virus type 1 in monkey cells. *J. Gen. Virol.* **76**, 2723–2730 (1995).
5. Himathongkham, S. & Luciw, P.A. Restriction of HIV-1 (Subtype B) replication at the early step in rhesus macaque cells. *Virology* **219**, 485–488 (1996).
6. Igarashi, T. *et al.* Protection of monkeys vaccinated with vpr and/or nef-defective simian immunodeficiency virus strain mac/human immunodeficiency virus type 1 chimeric viruses: a potential candidate live-attenuated human AIDS vaccine. *J. Gen. Virol.* **78**, 985–989 (1997).
7. Agy, M.B. *et al.* Infection of Macaca nemestrina by human immunodeficiency virus type-1. *Science* **103**, 103–106 (1992).

8. Shibata, R. *et al.* Isolation and characterization of a syncytium-inducing, macrophage/T-cell line-tropic human immunodeficiency virus type 1 isolate that readily infects chimpanzee cells *in vitro* and *in vivo*. *J. Virol.* **69**, 4453–4462 (1995).
9. Shibata, R. *et al.* Infection and Pathogenicity of Chimeric SIV/HIV Viruses in Macaques; Determinants of High Virus Loads and CD4 cell Killing. *J. Infect. Dis.* **176**, 362–373 (1997).
10. Li, J., Lord, C.I., Haseltine, W., Letvin, N.L. & Sodroski, J. Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. *J. AIDS.* **5**, 639–646 (1992).
11. Zilversmit, D.B., Boyd, G.A. & Brucer, M. The effect of particle size on blood clearance and tissue distribution of radioactive gold colloids. *J. Lab. Clin. Med.* **40**, 255–266 (1952).
12. Shibata, R. *et al.* Resistance of previously infected chimpanzees to successive challenges with a heterologous intraclade B strain of human immunodeficiency virus type 1. *J. Virol.* **70**, 4361–4369 (1996).
13. Brunner, K.T., Hurez, D., McCluskey, R.T. & Benacerraf, B. Blood clearance of P32-labeled vesicular stomatitis and Newcastle disease viruses by the reticuloendothelial system in mice. *J. Immunol.* **85**, 99–105 (1960).
14. Mims, C.A. Rift valley fever virus in mice. II. Adsorption and multiplication of virus. *Brit. J. Exp. Pathol.* **37**, 110–119 (1956).
15. Mims, C.A. The response of mice to large intravenous injections of Ectromelia virus II. The growth of virus in the liver. *Brit. J. Exp. Pathol.* **40**, 533–542 (1959).
16. Schultz, I. & Neva, F.A. Relationship between blood clearance and viruria after intravenous injection of mice and rats with bacteriophage and poliovirus. *J. Immunol.* **94**, 833–841 (1965).
17. Wei, X. *et al.* Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117–122 (1995).
18. Ho, D. *et al.* Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123–126 (1995).
19. Perelson, A. *et al.* HIV-1 Dynamics *in vivo*: Virion clearance rate, infected cell lifespan, and viral generation time. *Science* **271**, 1582–1586 (1996).
20. National Institutes of Health in *Guide for the Care and Use of Laboratory Animals*, revised ed. DHHS publication number NIH 85–23 (1985).
21. Naidu Y.M. *et al.* Characterization of infectious molecular clones of simian immunodeficiency virus (SIVmac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIVmac. *J. Virol.* **62**, 4691–4696 (1988).
22. Adachi A. *et al.* Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59**, 284–291(1986).
23. Shibata, R. *et al.* Reactivation of HIV Type 1 in chronically infected chimpanzees following xenostimulation with human cells or with pulses of corticosteroid. *Aids Res. Hum. Retroviruses.* **13**, 377–381 (1997).
24. Haigwood, N.L. *et al.* Passive immune globulin therapy in the SIV/macaque model: early intervention can alter disease profile. *Immunol. Lett.* **51**, 107–114 (1996).
25. Shibata, R., Siemon, C., Czajak, S.C., Desrosiers, R.C. & Martin, M.A. Live attenuated SIV vaccines elicit potent resistance against a challenge with an HIV-1 chimeric virus. *J. Virol.* **71**, 8141–8148 (1997).
26. Willey R.L. *et al. In vitro* mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. *J. Virol.* **62**, 139–147 (1988).
27. Reed, L.J. & Muench H. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 493–497 (1938).
28. Dewar, R.L. *et. al.* Application of branched DNA signal amplification to monitor human immunodeficiency virus type 1 burden in human plasma. *J. Infect. Dis.* **170**, 1172–1179 (1994).